*IN THE UNITED STATES PATENT AND TRADEMARK OFFICE*

Applicant: Ebrahim ZANDI, et al.

Title: COMPOSITION AND METHOD FOR RECONSTITUTING IkB KINASE IN YEAST AND METHODS OF USING SAME

Appl. No.: 10/079,949

Filing Date: 2/19/2002

Examiner: Prouty, Rebecca E.

Art Unit: 1652

Confirmation Number: 6542

**SUPPLEMENTAL DECLARATION UNDER 37 CFR SECTION 1.131**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. We, Ebrahim Zandi and Beth Schomer Miller, hereby declare as follows.

2. We are the Ebrahim Zandi and Beth Schomer Miller, who are named as co-inventors of the above-identified application.

3. That we conceived and reduced to practice in the United States the transformation of an IKK subunit gamma (γ) gene, an IKK subunit alpha (α) gene and/or an IKK subunit beta (β) gene into yeast and the separation from that yeast a substantially homogenous and biologically functional IKK protein complex prior to November 15, 2000, the online publication date of the literature article Li et al. (2001) "Role of IKKγ/NEMO in Assembly of the IκB Kinase Complex"

Journal of Biological Chemistry 276(6):4494-4500. Attached hereto is Exhibit A, a copy of pages from laboratory notebooks recorded by Beth Schomer Miller working under our direct control and supervision showing a reduction to practice wherein the activity of a purified IKK complex from yeast transformed with either IKKβ, IKKβγ, or IKKαβγ compared to mammalian IKK complex isolated from control Hela cells or TNF stimulated HELA cells was determined. These experimental results demonstrate that a yeast cell was transformed with an IKK subunit gamma (γ) gene, an IKK subunit alpha (α) gene and/or an IKK subunit beta (β) gene. The yeast was then grown and a substantially homogenous and biologically functional IKK protein complex was separated from the yeast.

4. That the documents in Exhibit A, which relates to the aforementioned actual reduction to practice, are exact and true copies. All personal information, including names and dates have been redacted from the documents, but all dates are prior to November 15, 2000.

5. Attached hereto is Exhibit B. Exhibit B is a typed version of Exhibit A and a true and exact representation of the handwritten information of Exhibit A.

6. As specifically identified on pages 1 and 9 of Exhibit B, the purpose of the experiment was to compare the activity of recombinantly produced IKK complexes that were isolated from yeast transformed with IKK subunits alpha, beta, and gamma (yαβγ), subunits beta and gamma (yβγ), subunit beta (yβ), to IKK complexes isolated from non-stimulated Hela cells (HNS) and TNF-stimulated Hela cells (TNF). The remaining pages set forth the experimental protocols, the resulting activity of the isolated IKK complexes and the amount of IKKβ subunit present in the isolated IKK complexes. The gels shown on pages 4, 5, 10 and 11 of Exhibit B demonstrate that the activity of recombinantly produced IKK complex isolated from yeast transformed with IKK subunits alpha, beta, and gamma (yαβγ) is higher than purified IKK complex from non-stimulated HeLa cells and the same or slightly higher than purified activated IKK complex from TNF-stimulated Hela cells. Similar gels and results are show in Figure 3 and on page 15, lines 21 to 27 of the above-identified application.

Atty. Dkt. No. 064189-0501

7. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are true; and further that all statements made herein are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United Sates Code, and that such false statements may jeopardize the validity of legal decisions of any nature based on them.

Respectfully submitted,

**Ebrahim Zandi**

Ebrahim Zandi 02-10-2009

Signature: Date:

**Beth Schomer Miller**

Signature: Date:

Atty. Dkt. No. 064189-0501

7. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are true; and further that all statements made herein are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United Sates Code, and that such false statements may jeopardize the validity of legal decisions of any nature based on them.

Respectfully submitted,

**Ebrahim Zandi**

_____ _____

Signature: Date:

**Beth Schomer Miller**

Beth Schomer Miller 10 Feb 09

Signature: Date:

# Exhibit A

Tops 36161
MADE IN U. S. A.

[redacted] Purpose: to compare IKK activity in
γ αβγ γδ γβ HNS TNF

Butan GF βδ 8.2% good HA signal in 2 sec exp.
SML Fr 10 or 11

Butan GF αβδ good α→γ 1min SML Fr 10-11
1) γ→βδ
HA good signal 15 sec similar to
1) of [redacted] γ→βγ Fr 10

Butan β HA detect some Fr 15 after 1min (15)

---

HNS GF [redacted]
HNS β detected in 10+11 after 1min 20)
TNF weakly detected in 10+11 40min 20)
INP. identical.

Hugo's westerns were also poor for detection of
β in Balance + αβγ in his assays.
Will have to play around with amounts

---

HNS Q20 + TNF Q20 were separated by gel filtration
INP. could detect ~~with~~ 5-15) by αIKK β + γ western
in 15 sec.

Less present than 5) γ IKK

Put fractions in gel filtration → ~10 fold dilution
would need to use 150) for same amt.
150(10) + 150(11)
Concentrate ~~300~~) → 30)

Use: 5.10.15

[redacted]

B-HA fraction 15 G.F.
3 + 12 1x KA
5 + 10 1x
10 + 5 1x
[redacted]

Bδ -HA Fr 10
3 + 12 1x
5 + 10 1x
10 + 5 1x
[redacted]

αBδ Fr 10-11
3 + 12 1x
5 + 10 1x

HNS Q 20 → Sp 6 GF 10-11 17 mw
200 + 200 → 40
use 5, 10, 15
+ 10 + 5 1x

TNF Q 20 → Sp 6 GF 10-11
200 + 200 → 40
use 5, 10, 15
+ 10 + 5 1x

Load 35 each

| | | |
|---|---|---|
| 1 | empty | |
| 2 | empty | |
| 3 | B3 | |
| 4 | 5 | |
| 5 | 10 | |
| 6 | Bδ 3 | |
| 7 | 5 | all |
| 8 | 10 | |
| 9 | αBδ 3 | |
| 10 | 5 | |
| 11 | HNS 5 | |
| 12 | 10 | |
| 13 | 5 | |
| 14 | TNF 5 | |
| 15 | 10 | |
| 16 | 5 | |

ULTAFREE MC
50, KD
put
300 1x Kinase buffer in bottom
↓
prevent drying
↑ 1 hr

Should recover
≤ 40 retentate
recover
25 HNS add 15 1x
40 TNF

1. Aliquot extract + buffer according to table
2. Add 30 Kinase Cocktail Inc 30' 30°C
3. Add [redacted] SDS PAGE, heat
4. Load 10 gel

Only G

Cocktail - 15
10x Kinase 45
20mM DTT 45
200μM ATP 45
0.5 ng/μL GST-Her 30
γ ATP 7.5 [redacted] 90-58
H2O [redacted] 277.5

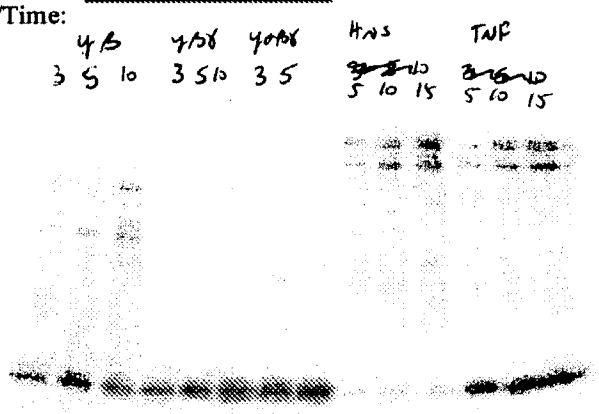
3 [redacted] 58

[redacted] Purpose: to compare activity of
γB vs γBγ vs γδBγ vs IHNS vs TNF

| 10% gel (10-10) | | Stack |
|---|---|---|
| 30% acryl | 5 ml | 1.05 |
| 8.8 | 3.75 | 1.9 (6.8) |
| $H_2O$ | 6.25 | 4.5 |
| APS | 200 100 | 75 |
| TEMED | 200 10 | 10 |

File/Range: D:\Users\1012bsm.gel / 0.000-45853 Counts / 1.000000
User Name: phospho
Image Name: D:\Users\1012bsm.gel
Image Comment: yeast b bg abg HNS TNF-Hela
scanned 9:13 am to2:05 pm

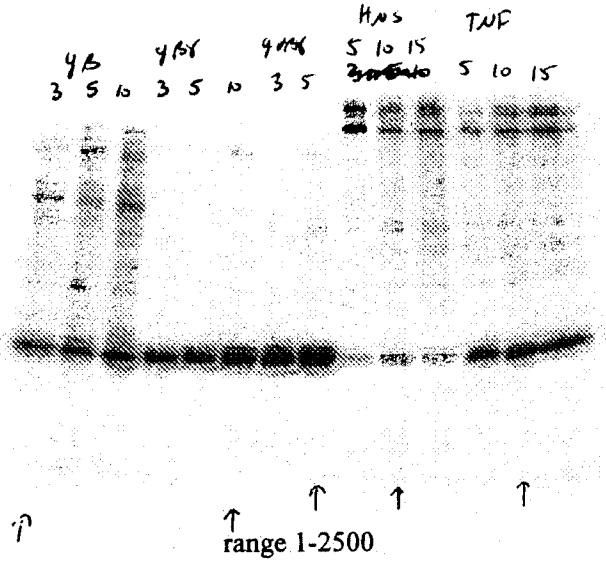
Present Date/Time
Scan Date/Time:
Prep. Date/Time:



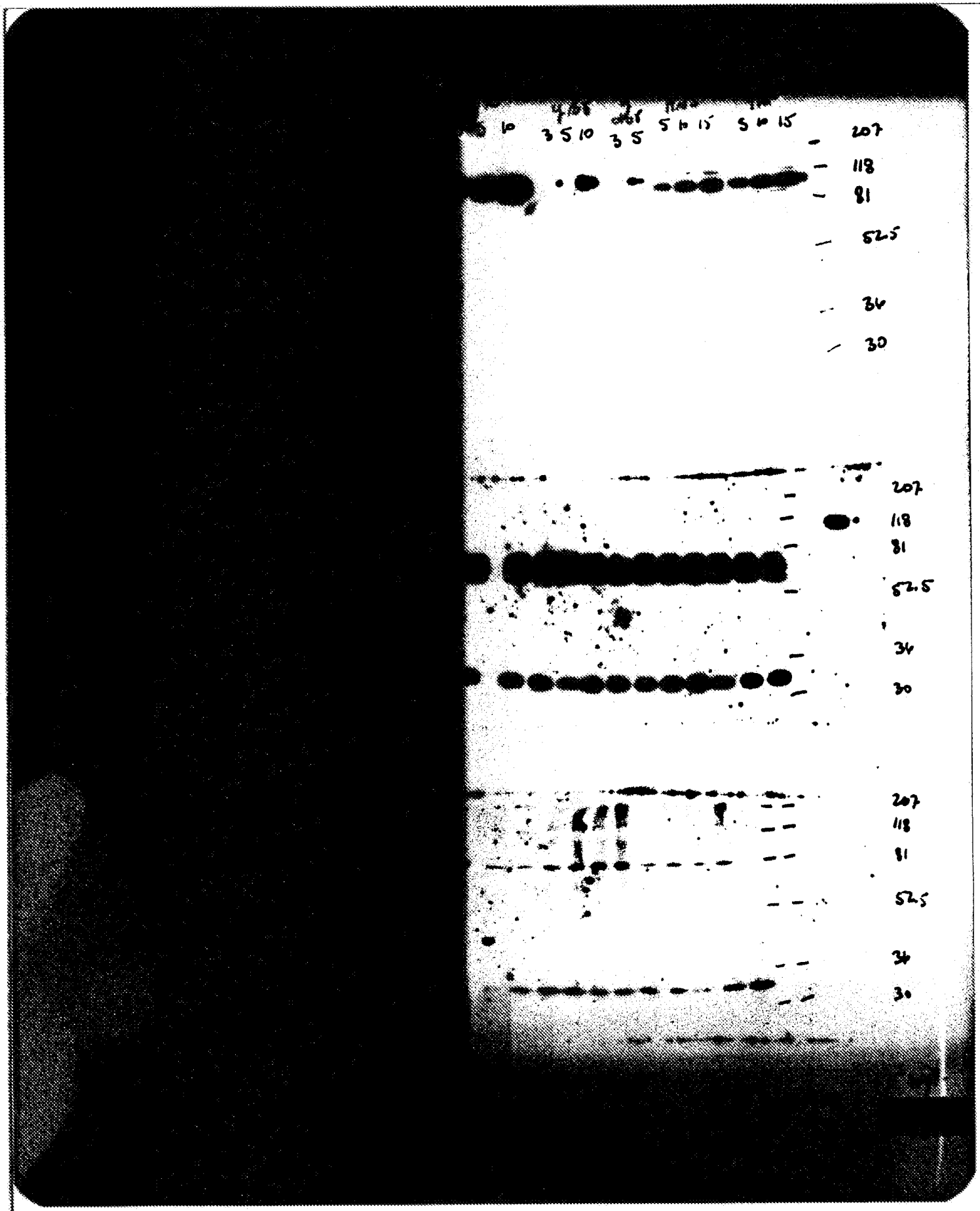

range 1-10,000

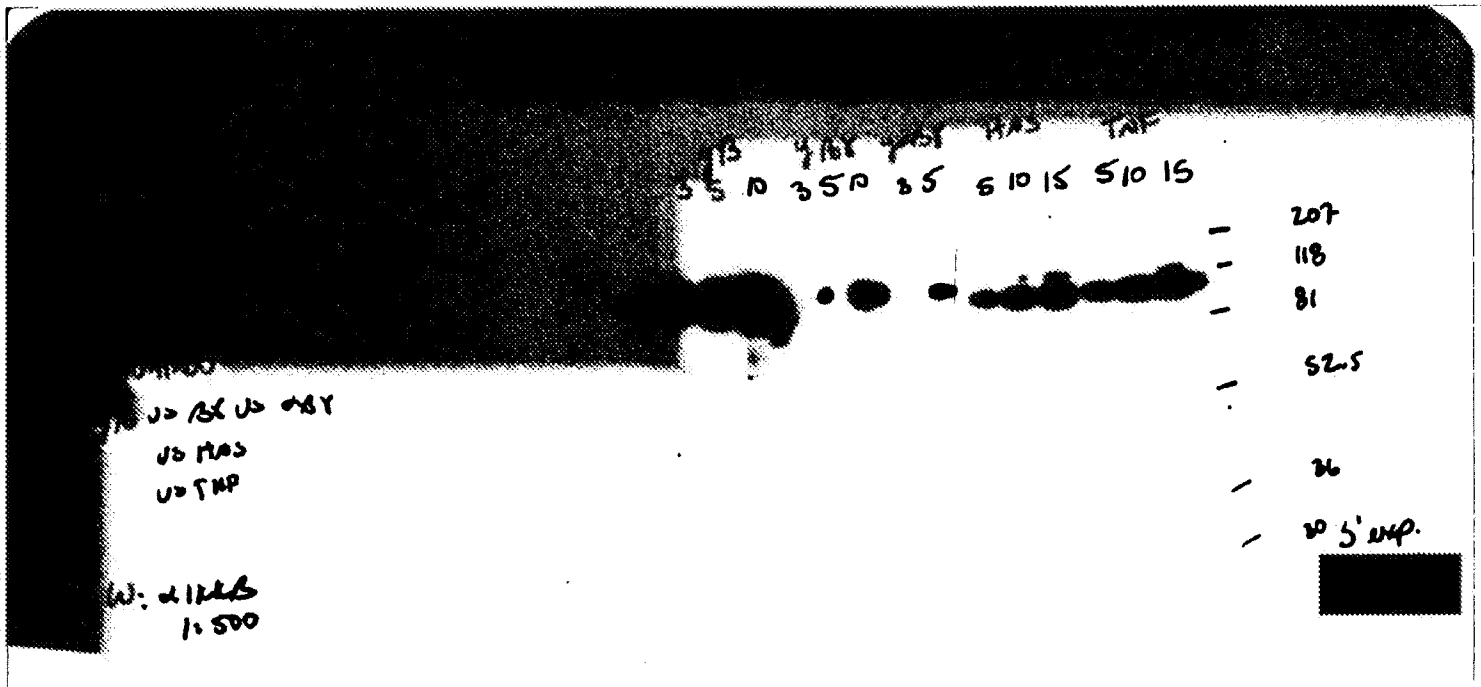
File/Range: D:\Users\1012bsm.gel / 0.000-45853 Counts / 1.000000
User Name: phospho
Image Name: D:\Users\1012bsm.gel
Image Comment: yeast b bg abg HNS TNF-Hela
scanned 9:13 am to2:05 pm

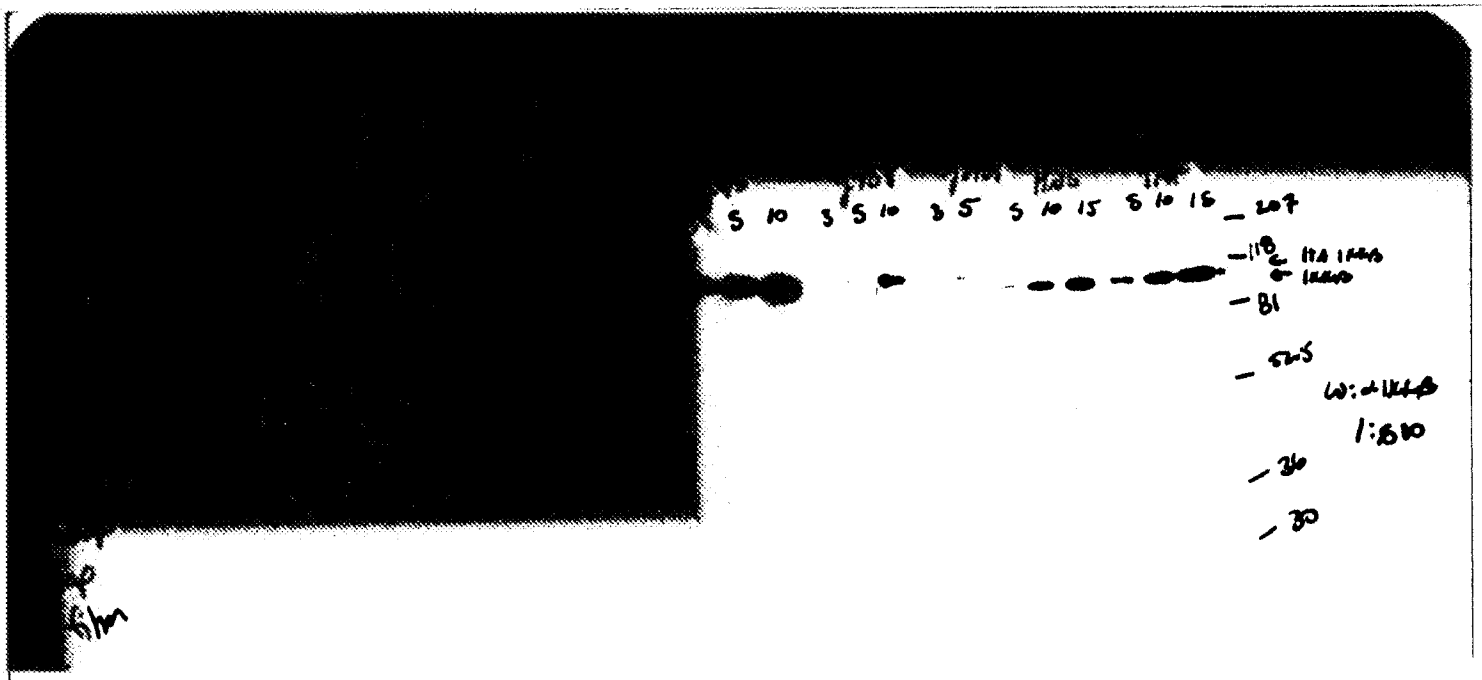
Present Date/Time: [redacted]
Scan Date/Time:
Prep. Date/Time:




range 1-2500


207
118
81
52.5
34
30
207
118
81
52.5
34
30
207
118
81
52.5
34
30


207
118
81
52.5
36
20 5' exp.


5 10 3 5 10 3 5 5 10 15 5 10 15
207
118
81
36
30
1:500

TOPS 36161
MADE IN U. S. A.

[redacted] Purpose: to compare IKK activity in
yA vs yBr vs. yaBr vs HNS vs TNF-Hela

repeat of 10-11 with attempt to use more similar amounts

HNS & TNF (Q20 → sup6 GF 10 + 11)
Put 300 1x IKinase buffer in bottom to prevent drying.
Top: 200 sup 6 GF 10 + 200 sup 6 GF 11

Recover ~ 40 + adjust vol. to 40 (1x IKA)

B-HA fraction 15

5 + 45 1x

| Tube/lane | | Dilute 1:10 in 1x Kinase | |
|---|---|---|---|
| 2 | 0.5 | 5 | + 16 1x |
| 3 | 1 | 10 | + 11 1x |
| 4 | 2 | 20 | + 1 1x |
| 5 BR - | 5 | 8 (Hela TNF) | + 14 1x |
| 6 | 14 | ~10 | + 7 1x |
| 7 | 21 | ~15 | +0 |
| 8 +BR | 7 | | + 14 1x |
| 9 | 14 | | +7 1x |
| 10 | 21 | | +0 |
| 11 HNS | 5 | | + 16 1x |
| 12 | 10 | | + 11 1x |
| 13 | 15 | | + 6 1x |
| 14 TNF | 5 | | + 16 1x |
| 15 | 10 | | + 11 1x |
| 16 | 15 | | + 6 1x |
| 17 mw | | | |

all loaded correctly / 40 each.

21
35
56

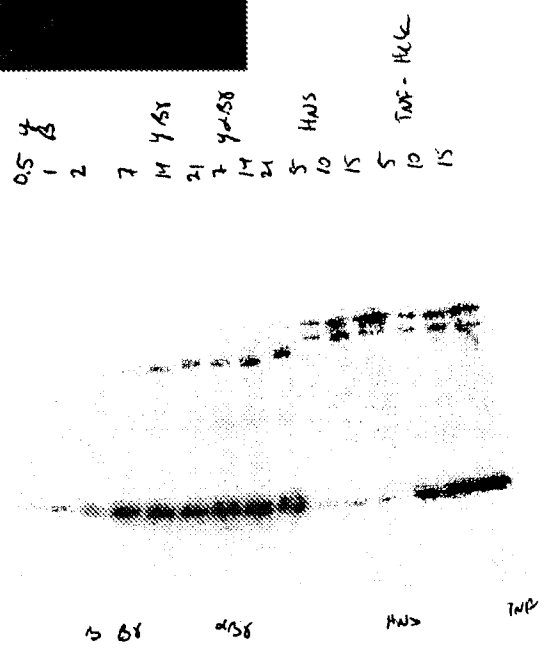
1. Aliquot extract + buffer 35
2. Add [redacted] Kinase Cocktail Inc 30' 30°C
3. Add [redacted] 6x SDS PAGE Heat 95°C 5'
4. Load 10% gel (40)

| Cocktail | 16 samples | + 4 |
|---|---|---|
| 10x Kinase | 48 | 12 |
| 20mM DTT | 48 | 12 |
| 200μM ATP | 48 | 12 |
| GST-IKKβ | 32 | 8 |
| $^{32}$P ATP | 8 (900-58) | 2 |
| $H_2O$ | 296 | 74 |
| | 480 | 120 |

1M DTT
20 mM DTT
.02 1M + .98 $H_2O$

File/Range: D:\Users\1017bsm.gel / 0.000-45853 Counts / 0.814331
User Name: phospho
Image Name: D:\Users\1017bsm.gel
Image Comment: 2 experiments
1. 3 M urea GF column fractions (concentrated)
2. yeast b, bg, abg, HNS, TNF stim Hela

Present Date/Time: [redacted]
Scan Date/Time:
Prep. Date/Time:

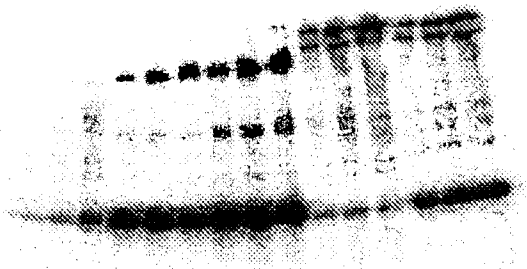



scale 1-2500

11 yaBS

File/Range: D:\Users\1017bsm.gel / 0.000-45853 Counts / 0.814331
User Name: phospho
Image Name: D:\Users\1017bsm.gel
Image Comment: 2 experiments
1. 3 M urea GF column fractions (concentrated)
2. yeast b, bg, abg, HNS, TNF stim Hela

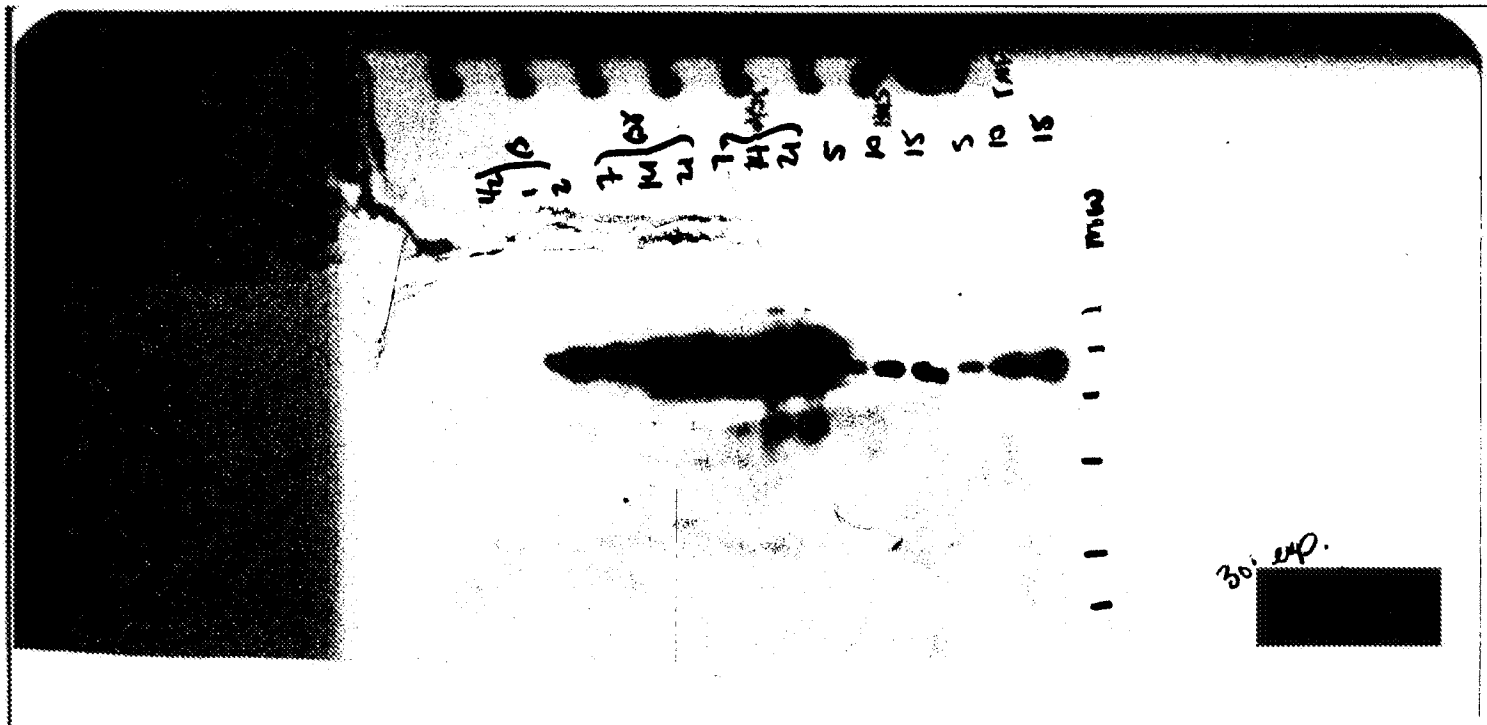
Present Date/Time: [redacted]
Scan Date/Time:
Prep. Date/Time:



scale 1-250

[redacted]

[redacted]


kDa
30' exp.

# Exhibit B

Purpose: to compare IKK activity in yαβγ yβγ yβ HNS TNF

Beth βγ 8-25 good HA signal in 2 sec exp.
GF 5μL Fr 10 or 11

Beth αβγ good α+γ 1 min 5μL Fr 10-11
GF 1λ yαβγ
HA good signal 15 sec similar to
1λ of _____ γ → βγ Fr 10

Beth βHA detect some Fr 15 after 1 min (15λ)

---

HNS GF
HNS β detected in 10 + 11 aft 1 min 20λ
TNF weakly detected in 10 + 11 40 min 20λ

INP identical

Hugo's westerns were also poor for detection of
β in β alone + αβγ in his assays.
I'll have to play around with amounts

---

HNS Q20 + TNF Q20 were separated by gel filtration
Inp. could detect 5 – 15λ by αIKK β + γ Western in 15 sec.

Less present than 5λ yIKK

Put fractions in gel filtration → ~ 10 fold dilution
would need to use 150λ for same amt.

Concentrate 150(10) + 150(11) λ → 30λ

use: 5 . 10 . 15

β-HA fraction 15 G.F.

3λ + 12λ 1x KA
5λ + 10λ 1x
10λ + 5λ 1x

βγ-HA Fr 10

3 + 12λ 1x
5 + 10λ 1x
10 + 5λ 1x

αβγ Fr 10-11

3 + 12λ 1x
5 + 10λ 1x

HNS Q 20→ sup 6 GF 10 + 11

200 + 200 →40λ
use 5, 10, 15λ
\+ +
10λ 5λ 1x

TNF Q 20 → sup 6 GF 10 + 11

200 + 200 →40λ
use 5, 10, 15λ
\+ +
10λ 5λ
1x 1x

Load 35λ each

1 empty
2. empty
3. β 3
4. 5
5. 10
6. βγ 3
7. 5 All correctly loaded
8. 10
9. αβγ 3
10. 5
11. HNS 5
12. 10
13. 15
14. TNF 5
15. 10
16. 15
17. mw ULTA FREE MC 30, KD

Put 300λ 1x kinase buffer in bottom
↓
prevent drying filter

Should recover ≤ 40λ retentate
recover 25λ HNS
add 15λ 1x 40λ TNF

1. Aliquot extract + buffer according to table
2. Add 30λ Kinase cocktail Inc 30' 30°C
3. Add 9λ 6x SDS PAGE, heat
4. Load 10% gel — Only 6λ added

Cocktail – 15

| | |
|---|---|
| 10x Kinase | 45 |
| 20mm DTT | 45 |
| 200μm ATP | 45 |

0.5 mg/μl Gst - IκBα 30λ

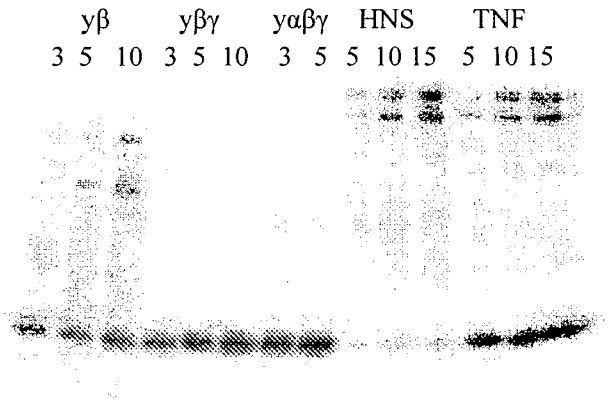
| | | |
|---|---|---|
| γ ATP | 7.5λ 906-58 | 3λ 906-57 |
| $H_2O$ | 277.5 | 4.5λ 58 |

Purpose: to compare activity of
yβ vs yβγ vs yαβγ vs HNS vs TNF

| 10% gel (10-10) | | Stack |
|---|---|---|
| 30% acryl | 5ml | 1.05 |
| 8.8 | 3.75 | 1.9 (6.8) |
| $H_2O$ | 6.25 | 4.5 |
| APS | 100 | 75 |
| TEMED | 10 | 10 |

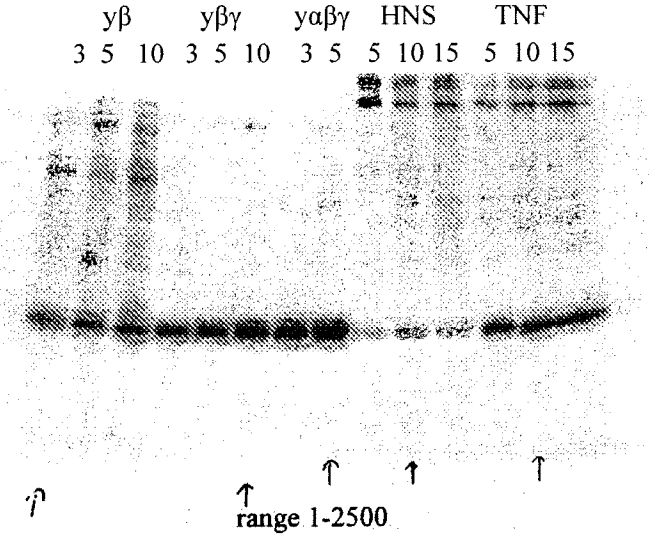
File/Range: D:\Users\1012bsm.gel / 0.000-45853 Counts / 1.000000
User Name: phospho
Image Name: D:\Users\1012bsm.gel
Image Comment: yeast b bg abg HNS TNF-Hela
scanned 9:13 am to2:05 pm

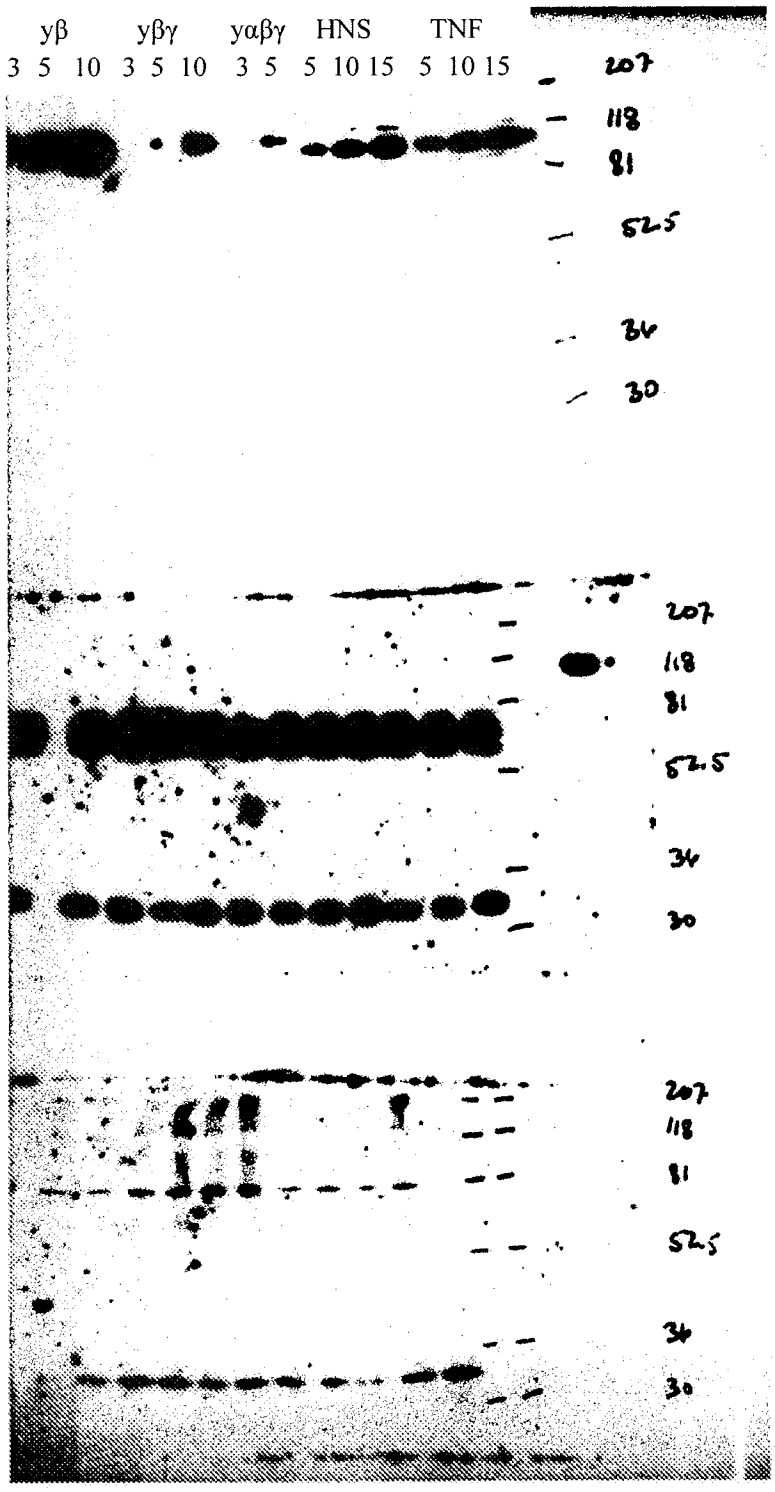
Present Date/Time: [redacted]
Scan Date/Time:
Prep. Date/Time:

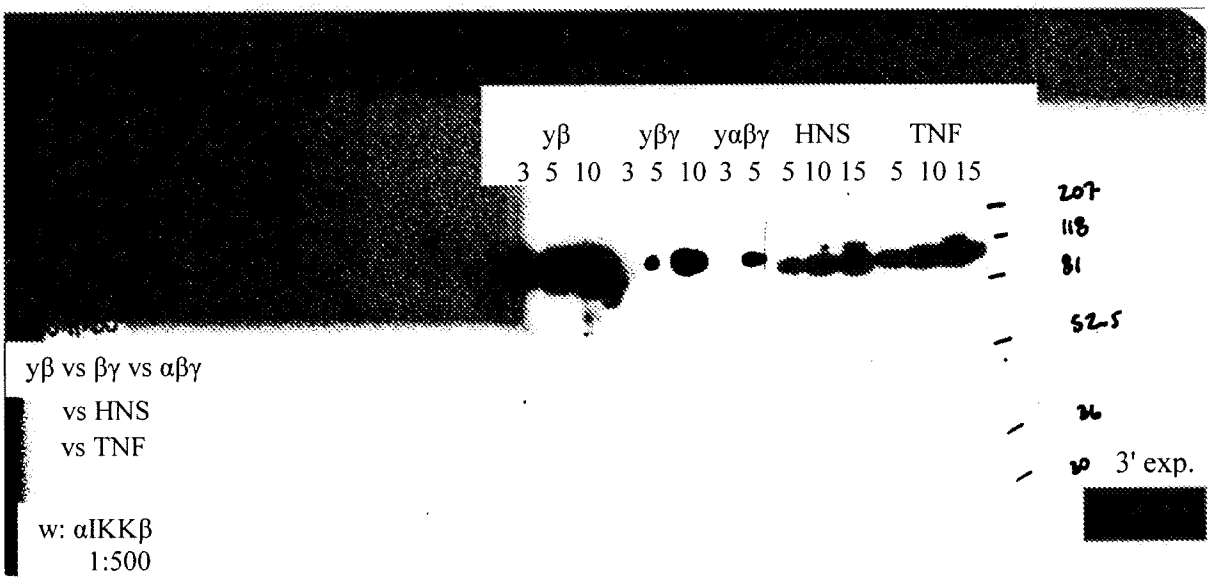


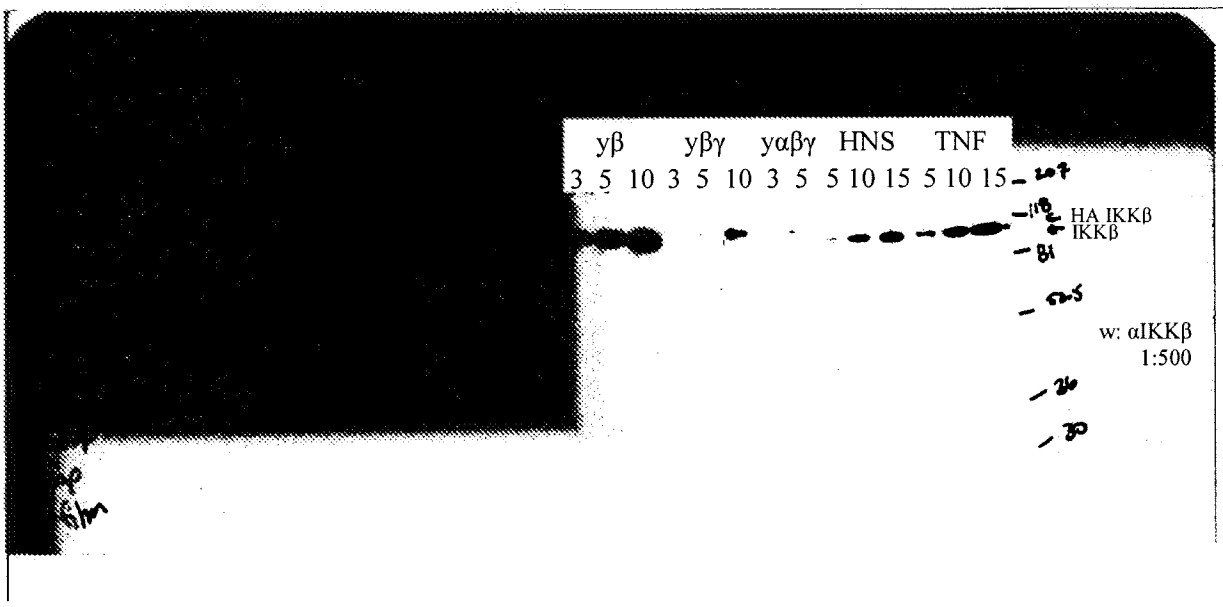

range 1-10,000

File/Range: D:\Users\1012bsm.gel / 0.000-45853 Counts / 1.000000
User Name: phospho
Image Name: D:\Users\1012bsm.gel
Image Comment: yeast b bg abg HNS TNF-Hela
scanned 9:13 am to2:05 pm
Present Date/Time: [redacted]
Scan Date/Time:
Prep. Date/Time:




yβ
yβγ
yαβγ
HNS
TNF
3 5 10 3 5 10 3 5 5 10 15 5 10 15
207
118
81
52.5
34
30


yβ yβγ yαβγ HNS TNF
3 5 10 3 5 10 3 5 5 10 15 5 10 15
207
118
81
52.5
36
28
3' exp.
yβ vs βγ vs αβγ
vs HNS
vs TNF
w: αIKKβ
1:500


yβ
yβγ
yαβγ
HNS
TNF
3 5 10 3 5 10 3 5 5 10 15 5 10 15
HA IKKβ
IKKβ
w: αIKKβ
1:500

Purpose: to compare IKK activity in
yβ vs yβγ vs yαβγ vs HNS vs TNF-Hela

repeat of 10-11 with attempt to use more similar amounts

HNS + TNF (Q20 → sup6 GF 10 + 11)
Put 300λ 1x 1 kinase buffer in bottom to prevent drying.
Top: 200λ sup6 GF 10 + 200λ sup6 GF 11

recover ~ 40λ + adjust vol. to 40λ
(1x KA)

β – HA fraction 15
5λ + 45λ 1x
Dilute 1:10 in 1x kinase

| Tube/lane | | | | |
|---|---|---|---|---|
| 2 | 0.5λ | 5λ + 16λ | 1x | |
| 3 | 1λ | 10λ + 11λ | 1x | |
| 4 | 2λ | 20λ + 1λ | 1x | |
| | | equiv. amount | | |
| 5 | βγ – 7λ | ~ 5λ (Hela TNF) | +14λ | 1x |
| 6 | 14λ | ~10 | +7λ | 1x |
| 7 | 21λ | ~15 | +0 | |
| 8 | αβγ 7 + 14λ | 1x | | |
| 9 | 14 + 7λ | 1x | | |
| 10 | 21 + 0 | | | |
| 11 | HNS 5 + 16λ | 1x | | |
| 12 | 10 + 11λ | 1x | | |
| 13 | 15 + 6λ | 1x | | |
| 14 | TNF 5 + 16λ | 1x | | |
| 15 | 10 + 11λ | 1x | | |
| 16 | 15 + 6λ | 1x | | |
| 17 | MW | | | |

21 all loaded correctly! 40λ each
35
56

1. Aliquot extract + buffer
2. Add 35λ kinase
   cocktail Jnc 30' 30° C
3. Add 12.2λ 6x
   SDS PAGE
   Heat 95° C 5'
4. Load 10% gel
   (40λ)

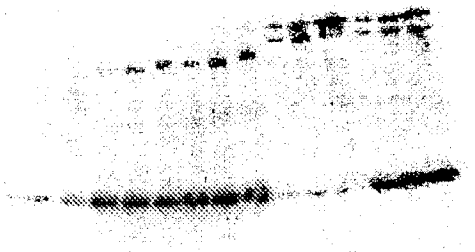
| Cocktail | 16 sample | + 4 |
|---|---|---|
| 10χ Kinase | 48λ | 12λ |
| 20mm DTT | 48λ | 12λ |
| 200μm ATP | 48λ | 12λ |
| GST – IKβα | 32λ | 8λ |
| $^{32}$P ATP | 8λ 906-58 | 2λ |
| $H_2O$ | 296 | 74 |
| | 480 | 120 |

20mm DTT
.02 m1 1M + .98m1 $H_2O$

File/Range: D:\Users\1017bsm.gel / 0.000-45853 Counts / 0.814331
User Name: phospho
Image Name: D:\Users\1017bsm.gel
Image Comment: 2 experiments
1. 3 M urea GF column fractions (concentrated)
2. yeast b, bg, abg, HNS, TNF stim Hela

Present Date/Time: [redacted]
Scan Date/Time:
Prep. Date/Time:

| yβ | | | yβγ | | | yαβγ | | | HNS | | | TNF-HeLa | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.5 | 1 | 2 | 7 | 14 | 21 | 7 | 14 | 21 | 5 | 10 | 15 | 5 | 10 | 15 |



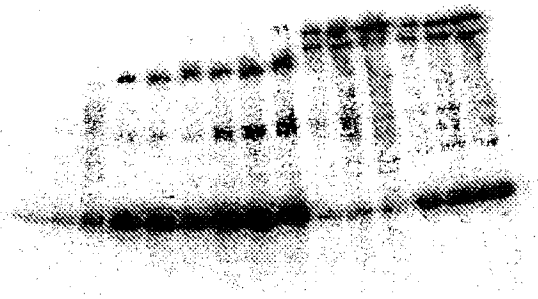
scale 1-2500

β , βγ αβγ HNS TNF

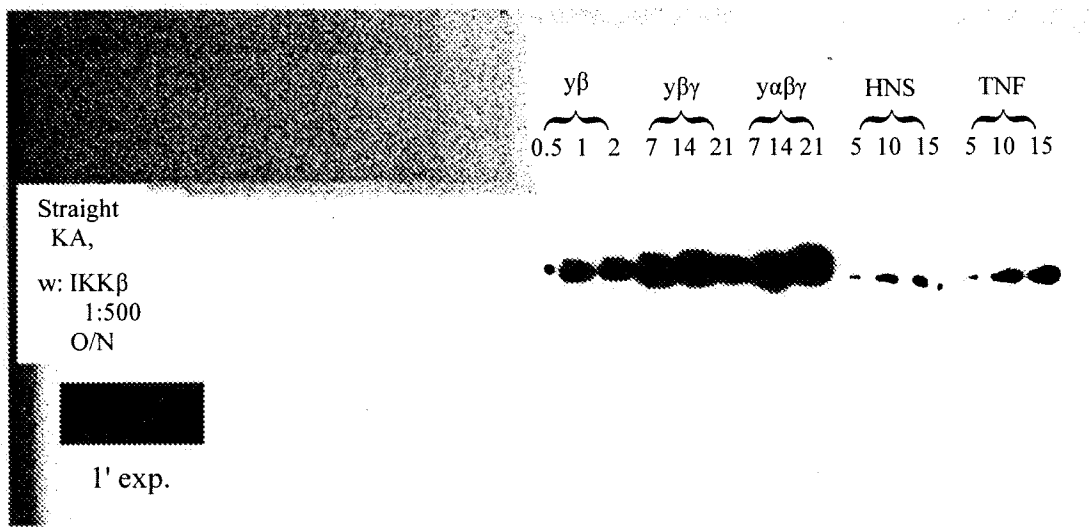
1λ yαβγ

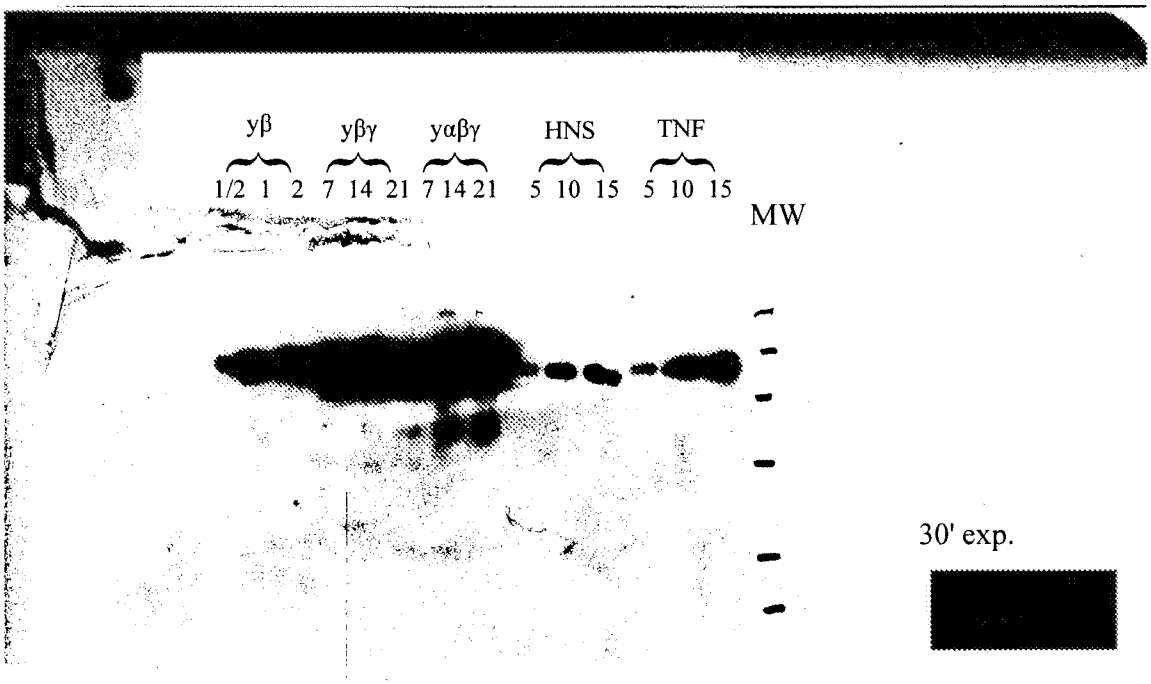
File/Range: D:\Users\1017bsm.gel / 0.000-45853 Counts / 0.814331
User Name: phospho
Image Name: D:\Users\1017bsm.gel
Image Comment: 2 experiments
1. 3 M urea GF column fractions (concentrated)
2. yeast b, bg, sbg, HNS, TNF stim Hela

Present Date/Time: [redacted]
Scan Date/Time:
Prep. Date/Time:



scale 1-250


yβ
yβγ
yαβγ
HNS
TNF
0.5 1 2
7 14 21
7 14 21
5 10 15
5 10 15
Straight
KA,
w: IKKβ
1:500
O/N
1' exp.


yβ
yβγ
yαβγ
HNS
TNF
1/2 1 2 7 14 21 7 14 21 5 10 15 5 10 15
MW
30' exp.